The Effect of $PGF_{2\alpha}$ on the Expression of Sodium Dependent Vitamin C Transporters in Early vs. Mid-Cycle Corpora Lutea of Sheep

Kaleen Percha

Advisor: Joseph S. Ottobre

Department of Animal Sciences, The Ohio State University

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Abstract

The corpus luteum (CL) is an ovarian structure responsible for progesterone secretion and the maintenance of early pregnancy. In the absence of pregnancy, the CL regresses in response to uterine prostaglandin $PGF_{2\alpha}$. Vitamin C is an antioxidant that scavenges free radicals generated during normal metabolic functions. Sodium dependent vitamin C transporters (SVCT) are responsible for maintaining high concentrations of vitamin C within the cell. Release of vitamin C from the CL is one of the first events to occur prior to luteolysis (regression). Exogenous $PGF_{2\alpha}$ causes a transient (less than 24h) depletion of vitamin C from CL in the early luteal phase (day 1-4 after estrus), and these CL do not regress. Exogenous $PGF_{2\alpha}$ in mid-luteal phase CL (day 10) causes an irreversible depletion of vitamin C, which is followed by luteal regression. Thus, the day 10 CL has acquired luteolytic capacity. The objectives of this research were to determine if exogenous $PGF_{2\alpha}$ affected the concentrations of mRNA for SVCT 1 & 2 in sheep corpora lutea, and to determine if the effect was dependent upon the luteolytic capacity of the CL. We also examined if there was an acute (2h) or sustained (24h) effect of $PGF_{2\alpha}$ on concentrations of SVCT mRNA. Mature ewes were randomly separated into two groups: early luteal phase (day 3) and mid luteal phase (day 10). Each group was further divided into two treatments, saline treated (control) or $PGF_{2\alpha}$ treated. From each animal, two CL were harvested, one at 2h and the other 24h after treatment. Using real time polymerase chain reaction (RT-PCR) we were able to estimate relative concentrations of SVCT mRNA within the cells. We found that SVCT1 did not amplify to measureable levels during standard curve validation while SVCT2 did. As such, only SVCT2 mRNA concentrations were analyzed in this study. We found that in early cycle CL, treatment with $PGF_{2\alpha}$ did not alter SVCT2 mRNA concentrations at 2 or 24hrs post treatment. As the CL aged from the early cycle to the mid luteal cycle, SVCT2

mRNA concentrations increased significantly within the corpus luteum. Finally, in mid cycle CL, SVCT2 mRNA concentrations did not change 2hrs after treatment with $PGF_{2\alpha}$, but decreased sharply 24hrs after treatment. Maintenance of SVCT2 mRNA concentrations in early cycle CL is likely critical in enabling those cells to recover their initial vitamin C concentrations 24hrs after treatment with $PGF_{2\alpha}$. The significant increase in vitamin C concentrations as the CL ages from the early cycle to the mid cycle, corresponds to an increase in transporter mRNA. With the increase in membrane transporters, the cell is able to take in more vitamin C which would account for the increased vitamin C concentrations seen in the mid cycle corpora lutea. The inability of mid cycle CL to recover their vitamin C concentrations 24hrs after treatment with $PGF_{2\alpha}$, is also related to the decrease in SVCT2 mRNA seen in these cells, because there were fewer transporters available with which to replenish cellular vitamin C concentrations. As we continue to increase our knowledge of the mechanisms involved in luteal function, we come closer to developing new reproductive techniques that can benefit both animals and humans in the future.

Introduction

The ovary experiences some of the greatest morphological changes in its shape and size than nearly any other organ in the female body. These changes are brought about as a result of the ever changing concentrations of hormones within the body, especially the hormones FSH (follicle stimulating hormone), and LH (luteinizing hormone), both of which are produced in the anterior pituitary gland. These hormones are responsible for stimulating follicular growth in the ovary, as well initiating ovulation. Progesterone is another critical hormone within the female body and is produced by the corpus luteum, an ovarian structure that forms from the ruptured follicle. Progesterone is responsible for keeping the uterus quiescent so that embryo implantation can occur and is also responsible for the maintenance of pregnancy. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is a hormone that is produced in the uterus and is responsible for triggering luteal regression in the absence of a pregnancy. Together these hormones are responsible for the dynamic changes that occur in the ovary during follicular development and maturation, and the subsequent formation and destruction of the resulting corpus luteum (Senger, 2005).

It has long been known that the breakdown of the corpus luteum (luteolysis) is accompanied by the generation of numerous free radical species, including superoxide and peroxide radicals (Sawada & Carlson, 1989). Free radicals can damage cellular and organelle membranes, lipids, DNA, carbohydrates and proteins (Luck, Jeyaseelan, & Scholes 1995). As such, the role of antioxidants to halt free radical damage within the ovary has been one of great interest. Vitamin C (ascorbic acid) is an antioxidant that is found in high concentrations within the corpus luteum and is thought to play a critical role in controlling the free radical populations generated within the corpus luteum prior to luteolysis. In addition to its antioxidant role, vitamin C is also known to act as an enzyme cofactor in collagen synthesis reactions (Luck, Jeyaseelan,

& Scholes 1995). During the estrous cycle the ovarian tissues are in a near constant state of change, and the cofactor role of vitamin C in collagen synthesis could be important in facilitating these changes.

Research has shown that concentrations of vitamin C found within the corpus luteum, increased during luteal formation and maturation, then decreased during luteal regression (Petroff, Dabrowski, Ciereszko, & Ottobre, 1997). Petroff et al. (1998) showed that injection of $PGF_{2\alpha}$ into mid to late porcine corpora lutea caused a rapid depletion of vitamin C into the ovarian venous drainage. Since depletion of luteal ascorbate was an early change in response to $PGF_{2\alpha}$ they speculated that this depletion could be a component in the sequence of events leading to luteal regression. Tsai and Wiltbank (1998) used a model to try and identify events induced by $PGF_{2\alpha}$ that were associated with luteal regression. They found that $PGF_{2\alpha}$ does not cause regression in the early luteal phase, but does cause regression in the mid to late luteal phase. In their model they investigated various responses to $PGF_{2\alpha}$ in the early vs. late luteal phase, including luteal vitamin C. They found that vitamin C concentrations decreased in response to $PGF_{2\alpha}$ at both early and mid luteal phases.

Recent research however, shows that in early corpora lutea (day 3), treatment with $PGF_{2\alpha}$ causes a decrease in vitamin C concentrations 2 hours after treatment, but shows that these corpora lutea are able to regain their initial vitamin C concentrations within 24 hours. Luteal vitamin C concentrations increased from early to mid cycle (day 10), suggesting that vitamin C could play important roles as corpora lutea mature and draw closer to the time of luteolysis. Treatment of sheep with $PGF_{2\alpha}$ during mid cycle caused a similar decrease in luteal vitamin C at 2 hours, but unlike early cycle corpora lutea, these cells were unable to recover their vitamin C concentrations after 24 hours. (Ottobre, unpublished).

Sodium dependent vitamin C transporters (SVCT) are active transporter proteins bound within the cell membrane that are responsible for maintaining high cellular concentrations of vitamin C. There are two known sodium dependent vitamin C transporters, SVCT1 and SVCT2. Recently, the DNA sequence that codes for these two vitamin C transporters was partially sequenced in sheep (Ceddia, 2005).

The objectives of this research were to determine if exogenous $PGF_{2\alpha}$ affects the concentrations of mRNA for sodium dependent vitamin C transporters (SVCT) 1 & 2 in sheep corpora lutea, and to determine if the effect is dependent upon whether the corpus luteum has, or has not, acquired luteolytic capacity. We also examined if there was an acute (2 hr) or a sustained (24 hr) effect of $PGF_{2\alpha}$ on concentrations of SVCT mRNA.

Materials and Methods

Experimental Design and Collection of Corpora Lutea:

Mature ewes (n=18) were obtained from the Ohio State University Sheep facility for use in this study. The experimental protocol and sheep used in this study were approved for use by The Ohio State Institutional Animal Care and Use Committee. Ewes were randomly divided into two groups, early luteal phase (day 3) and mid luteal phase (day 10). Ewes were then further divided into one of two treatment groups, treatment with exogenous $PGF_{2\alpha}$ or treatment with saline as a control. Two corpora lutea were removed from each animal. The first one was removed at 2h and the second at 24h post

treatment (Fig. 1)

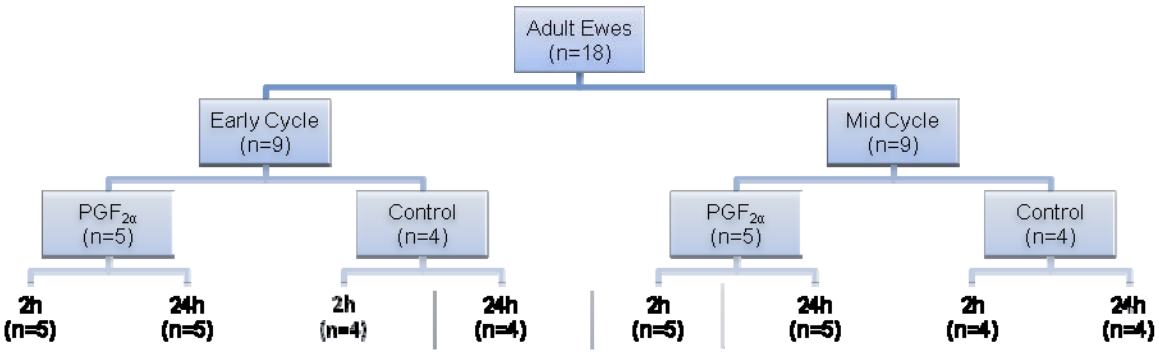

Figure 1. Experimental Design

The onset of estrus (day 0) was determined by twice daily observation in the presence of a vasectomized ram. On the assigned day of treatment (day 3 or day 10), animals were transported to the surgical facility. Ewes received intramuscular injections of $PGF_{2\alpha}$ (25mg dose, Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) or saline (time 0). Ewes were sedated using pentothal. The plane of anesthesia was maintained throughout the procedure using halothane

gas. A mid ventral laparotomy was preformed to expose the reproductive tract. The first corpus luteum was removed 2h post-treatment. A second corpus luteum was removed 24h post-treatment. Luteal tissues were immediately snap frozen in liquid nitrogen. Samples were stored at -80°C.

RNA Isolation and Purification:

RNA was isolated and purified from each corpora lutea using RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). RNA purity and integrity were determined from the absorbance ratio at 260 nm and 280 nm measured using the NanoDrop (ND-1000, Wilmington, DE). An endogenous reference gene, β-actin, was used to standardize the real time PCR runs.

Primer Design and Validation:

Primers for SVCT1 and SVCT2 were designed using the partial DNA sequences determined in our lab (Ceddia, 2005). The DNA sequences were entered into the Primer Express Software Version 3.0 (Applied Biosystems, Foster City, CA) and forward and reverse RNA primer sequences were generated (Table 1). Primers for ovine β-actin were designed using RNA primer sequences created by Laura Arborgast.

Table 1. Primer sequences for SVCT1, SVCT2, and ovine β-actin.

	SVCT1	SVCT2	Ovine β-actin
Forward (5' to 3')	CCTCCACCATACTGGACACCAT	TGGATAACACCATCCCAGGT	GCGCCCCCTGAGCGCAAG
Reverse (5' to 3')	TGGCACCCACGGATACG	GCCATCGAGAGACTTGCT	CATCTGCTGGAAGGTGGACA

Amplicon lengths for each set of primers were determined from the Primer Express Software. Primers for SVCT1, SVCT2 and ovine β-actin were tested for specificity by reverse

transcribing and amplifying RNA from the control luteal tissue using the SuperScript One-Step RT-PCR Kit (InVitrogen, Carlsbad, CA). Products were assessed using gel electrophoresis and run on a 10% tris-boric acid with EDTA (TBE) polyacrilamide gel (Bio-Rad Laboratories, Hercules, CA). Following the run, the polyacrilamide gel was stained using SYBR® Gold Nucleic Acid Gel Stain (InVitrogen, Eugene, OR) then visualized using UV light. Band lengths were determined using a 25bp DNA ladder (InVitrogen, Carlsbad, CA), then compared to the expected amplicon lengths determined by the primer software. Observation of band lengths that matched the expected amplicon length suggested that we amplified the desired product. If only one band was visible in each lane, it was determined that the binding of the primer was specific to the target RNA, no primer dimers had formed, and those primers could then be used to validate real time PCR.

Real Time PCR Validation:

SYBR Green real time PCR (RT-PCR) was used to relatively quantify levels of message for SVCT1, SVCT2 and β-actin present in each sample. SYBR Green is a method for RT-PCR that uses fluorescent molecules to determine the relative amounts of product present within a sample. SYBR Green is a non-specific florescent dye that intercalates into double stranded DNA and fluoresces. Being non-specific, SYBR Green will fluoresce with any double stranded DNA, including primer dimers, so it is imperative to ensure the binding specificity of the RNA primers prior to beginning RT-PCR. As amplification of the product occurs during PCR, more double stranded DNA is produced each cycle, causing a proportional increase in fluorescence. Sample mRNA concentrations can be determined by measuring the number of cycles required to reach a constant threshold level of fluorescence.

Before RT-PCR could begin, validation steps were needed to determine appropriate concentrations of primer and RNA. The first validation was to run a primer matrix to determine the most effective concentration of primer to use in each sample. Concentrations of 900nM, 300nM, and 100nM of each primer set (SVCT1, SVCT2, and β-actin) were run, and then amplification plots were analyzed. It was determined that primers at a concentration of 300nM would be most effective for the real time runs.

The second validation step was to run a standard curve to determine the appropriate concentration of RNA to include in each sample and to ensure that amplification was consistent. Each primer set had its RNA concentrations serially diluted in tenfold increments: 200ng, 20ng, 2ng, 0.2ng, 0.02ng, and 0.002ng. Each concentration was run in triplicate. Amplification was observed for each concentration, as were threshold values. Because the dilutions were performed in consistent tenfold increments, threshold values should be evenly spaced for each valid dilution. More specifically, for a standard curve to be valid, even spacing of amplification along the threshold needs to be seen for at least three dilutions (Fig. 2). Even spacing and precise triplicates were observed from 200ng RNA through 0.2ng RNA. We decided to use 20ng of RNA for this work. This would conserve the supply of RNA while preserving a reasonably fast amplification time (i.e., low C_T).

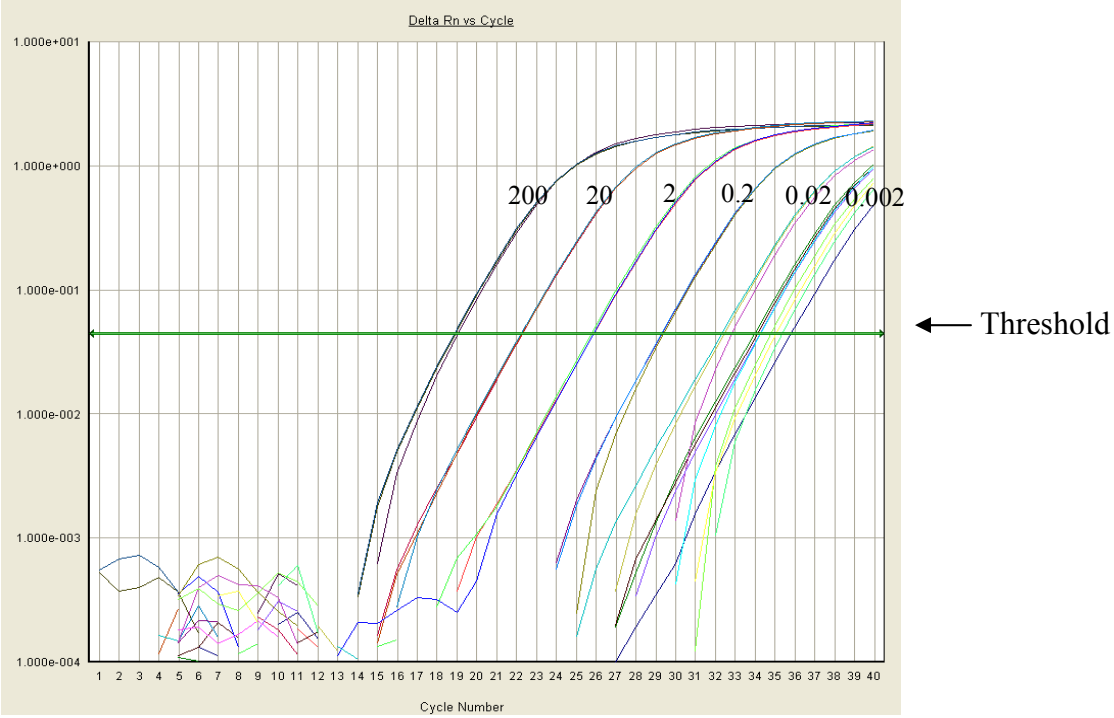

Figure 2. Standard Curve Study for SVCT2,

Amplification using 200, 20, 2, 0.2, 0.02, and 0.002 ng of RNA.

The final validation step for RT-PCR is to analyze the disassociation or melting curve. After the amplification cycles, a melting stage was added onto the end of each run. This melting stage causes all double stranded DNA present within the sample to disassociate, and the temperature at which this disassociation occurs is recorded. If there were multiple products present within the sample, such as primer dimers or other RNA contaminants, multiple disassociation curves at several different melting temperatures would be visible on the plot. Multiple curves would arise because different products melt at different temperatures, as

determined by the number of C-G and hydrogen bonds present within the DNA base pairs. Small products such as primer dimers, would disassociate at a lower temperature than the larger amplified product causing a 'shoulder' to appear early in the disassociation curve (Fig. 3). Only those disassociation curves with one peak, denoting only one amplified product could be validated (Fig. 4)

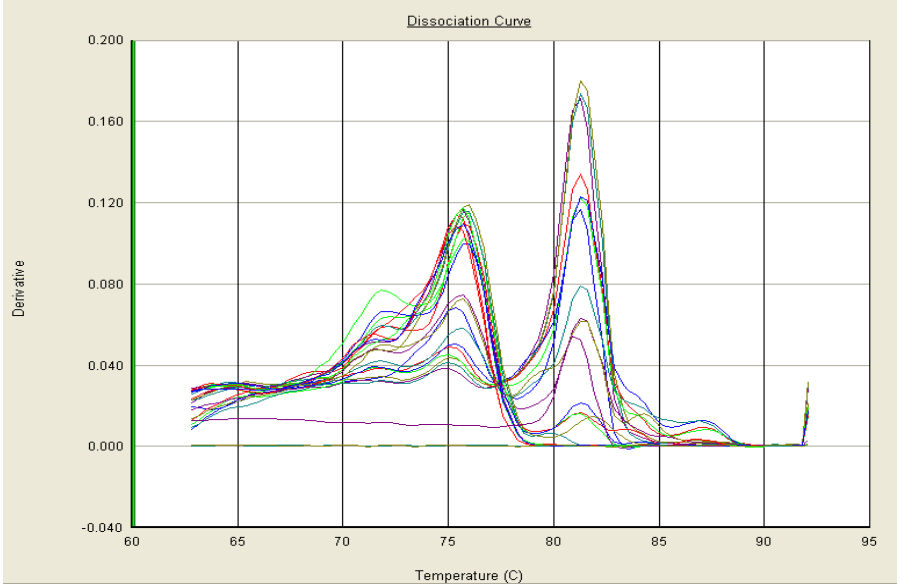

Figure 3. Disassociation Curve with Primer Dimers and other Amplification Products

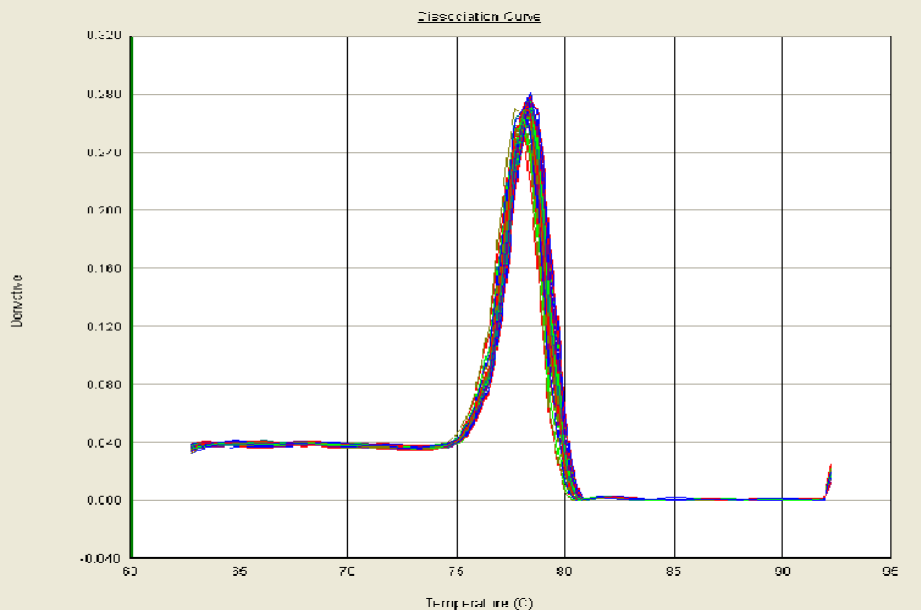

Figure 4. Disassociation Curve with No Primer Dimers or Other Amplification Products

Real Time PCR Analysis

Once the primers had been validated using RT-PCR, the samples from each of the four treatment groups were randomly selected and run in triplicate on 96-well plates. Sample RNA was run with the SVCT primers as well as the endogenous control β-actin primers. Power SYBR® Green RNA-to-C_T™ 1-Step Kit (Applied Biosystems, Foster City, CA) was used to prepare all samples according to the manufacturer's instructions with 25µL equaling the total reaction volume in each well. In addition to the samples, each plate also contained triplicate wells dedicated to RNA from a corpus luteum belonging to the control sheep used for all previous validation steps. This control sample was used to monitor consistency across plates.

Plates also contained triplicate control wells containing either no reverse transcriptase or no template RNA to ensure that the experiment was preformed correctly and that no contamination had occurred during plate preparation.

Samples were run using the ABI 7900 Real Time PCR system (ABI, Columbia, MD). Samples underwent 40 amplification cycles and afterwards each plate had an amplification plot and disassociation curve generated, that was then analyzed.

Data Expression and Statistical Analysis:

Relative concentrations of mRNA for SVCT were determined using the $\Delta\Delta C_T$ method. C_T is defined as the number of PCR cycles that it takes to meet a threshold level of fluorescence. The sample C_T for SVCT was first standardized using the C_T for β-actin (i.e., the first ΔC_T). Then, the data for individual samples was expressed as a fold change (second ΔC_T) from the mean standardized C_T for the control CL collected on day 3 at 2h.

Fold change data were analyzed using Proc Mixed procedure of SAS.

Results

We found that SVCT1 mRNA did not amplify to measurable levels during standard curve validation (Fig. 5).

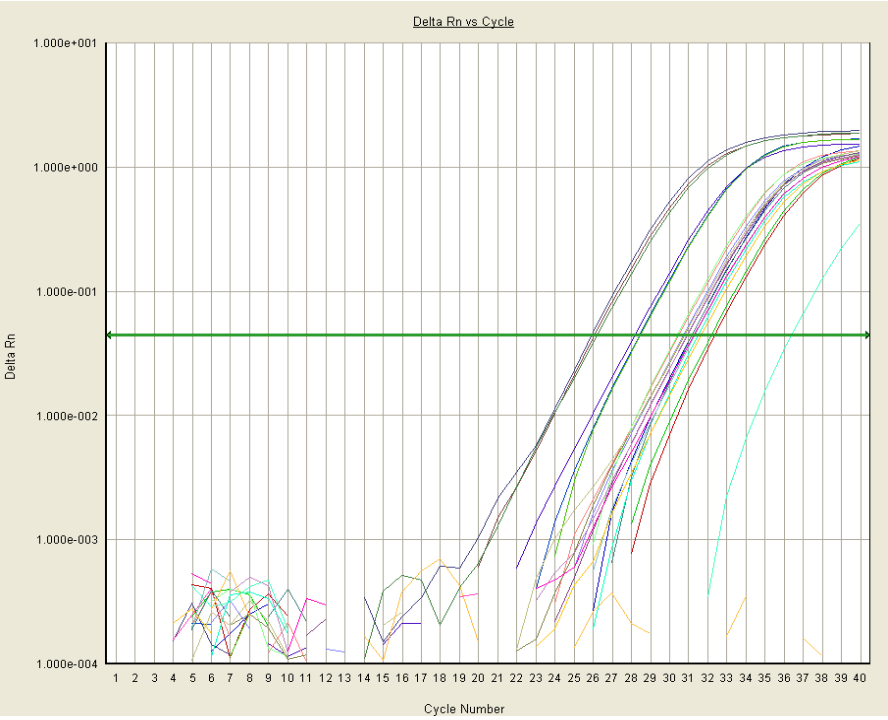

Figure 5. Standard Curve Study for SVCT1.

Amplification using 200, 20, 2, 0.2, 0.02, and 0.002 ng of RNA

To be a valid RT-PCR standard curve, amplification needs to be consistent for at least three different serial dilutions. For SVCT1, initial amplification at 200 ng did not begin until around cycle 26 of 40. Because amplification began so late, SVCT1 did not obtain the 3 linear threshold values necessary for standard curve validation. This late cycle amplification with such a high concentration of RNA suggests that there was limited SVCT1 message present within the corpora lutea available for amplification. According to Tsukagucki et al., (1999) certain tissues within the body are preferentially biased toward either SVCT1 or SVCT2. He found that endocrine tissues such as the pituitary gland, pancreas, testis, and adrenal gland are primarily

biased toward SVCT2. Tissues such as the kidney, liver and lung were biased toward SVCT1. From these data we speculate that the corpora lutea, as a known endocrine tissue, is biased toward SVCT2. Future tests using a known SVCT1 biased tissue will be needed to confirm that we can amplify SVCT1 message in tissues where it is prevalent. Because the SVCT1 primers could not be validated using RT-PCR, the experiment proceeded using only the ovine β-actin and SVCT2 primer sequences.

Once all samples had been run using RT-PCR, the data was analyzed using the ProcMixed procedure of SAS. We found that in the early luteal phase, $PGF_{2\alpha}$ treatment, and the hour in which the corpus luteum was collected, had no effect on the concentration of SVCT2 mRNA (Fig. 6). There was however, a significant increase in the concentration of SVCT2 message as the corpus luteum aged from the early cycle to the mid cycle ($p< 0.01$). $PGF_{2\alpha}$ had no effect on the SVCT2 message concentrations in mid cycle corpora lutea 2 hours after treatment. But, we found that it tended to decrease the concentration of SVCT2 mRNA message 24 hours after treatment with $PGF_{2\alpha}$ ($p= 0.06$).

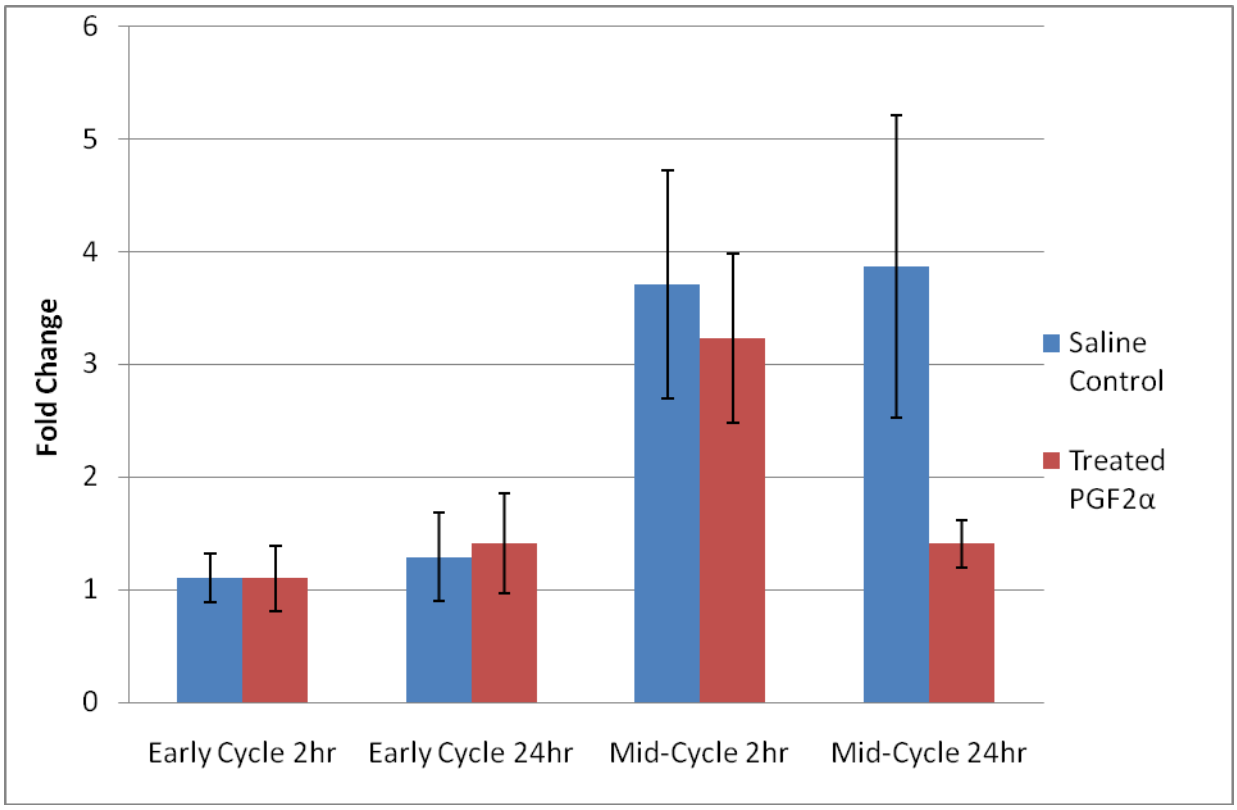

Figure 6. Fold change in SVCT2 mRNA in corpora lutea standardized using β-actin.

Values represent means +/- standard error. Data are from n=4 sheep for each control group and n=5 for each $PGF_{2\alpha}$ treated group. In each group, one corpus luteum was removed 2 hours after treatment, while a second corpus luteum was removed 24 hours after treatment.

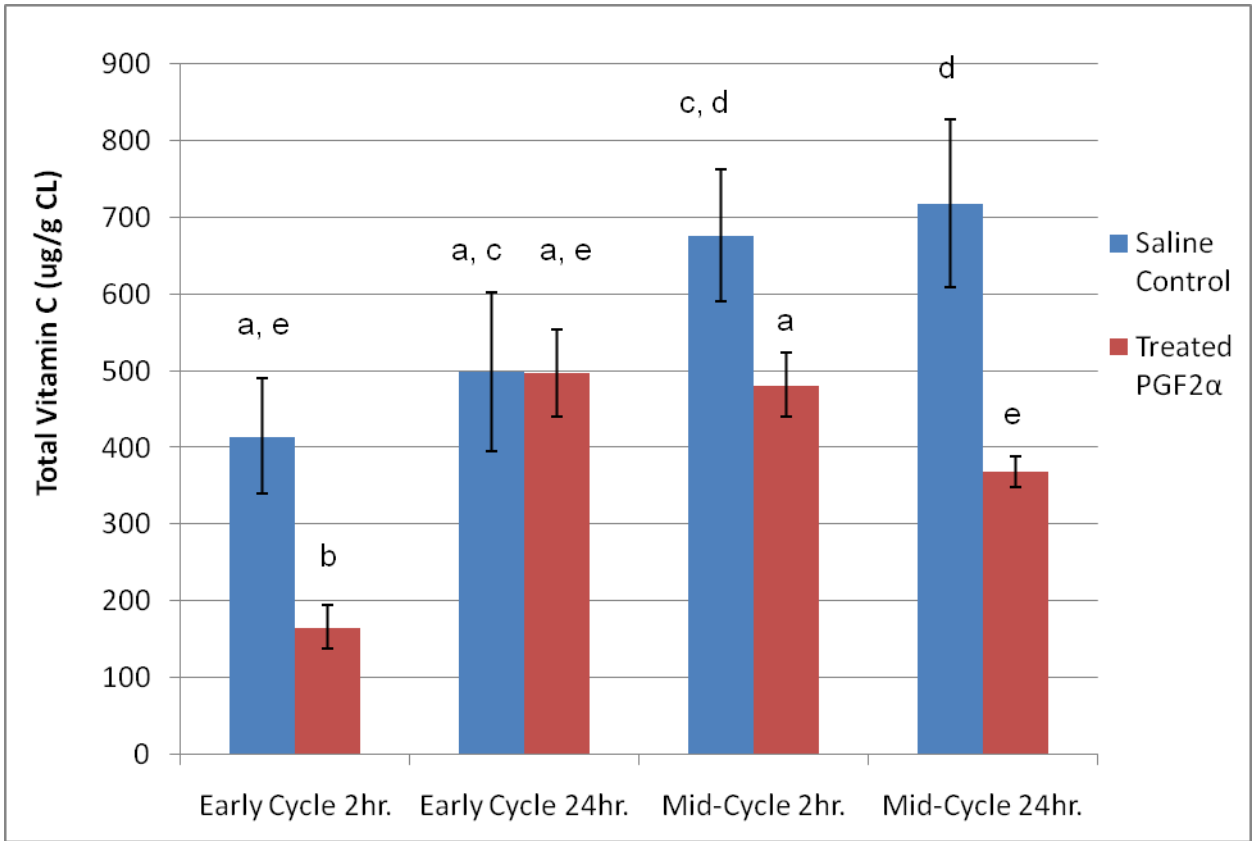

Figure 7. Vitamin C Concentrations in Sheep Corpora Lutea

Values represent means +/- standard error. Data are from a previous study using the same experimental design. Bars with uncommon letters are significantly different ($p < 0.05$).

Discussion

As shown in an earlier study in our laboratory, during the early luteal phase, vitamin C concentrations decreased significantly 2 hours after treatment with $PGF_{2\alpha}$, then recovered to pre-treatment levels within 24 hours (Fig. 7). SVCT2 mRNA concentrations remained constant in early luteal phase sheep corpora lutea after treatment with $PGF_{2\alpha}$ at both 2 and 24 hours.

At the start of the mid luteal phase, vitamin C concentrations within the corpora lutea were significantly higher than they had been in the early luteal phase. During the mid luteal phase, vitamin C concentrations decreased significantly 2 hours following treatment with $PGF_{2\alpha}$, and were unable to recover to pre-treatment levels within 24 hours. SVCT2 mRNA concentrations increased significantly from the early to the mid luteal phase. There was no change in SVCT2 mRNA concentrations 2 hours after treatment with $PGF_{2\alpha}$, but SVCT2 mRNA concentrations had plummeted 24 hours post treatment.

Vitamin C concentrations increased significantly from the early to the mid luteal phase. This is consistent with the increase in SVCT2 mRNA concentrations as the corpus luteum ages, because an increase in active sodium dependent transporters would allow the luteal cells to take in more vitamin C. In the early luteal phase, $PGF_{2\alpha}$ did not alter SVCT2 mRNA concentrations, which may have permitted the recovery of vitamin C concentrations within 24 hours of treatment with $PGF_{2\alpha}$. In the mid luteal phase, the decrease in SVCT2 mRNA concentrations 24 hours after treatment with $PGF_{2\alpha}$ most likely accounts for the sustained loss of luteal vitamin C concentrations and may be a factor in acquisition of luteolytic capacity.

As more is learned about the cause and effect relationship between vitamin C concentrations and the amount of SVCT2 mRNA present within the corpus luteum, a better understanding of the role vitamin C plays in the maintenance of the corpus luteum is gained. As

we continue to increase our knowledge of the mechanisms involved in luteal development, maintenance, and regression, we come closer to developing new reproductive techniques that could benefit both animals and humans in the future.

As we expand our knowledge of luteal function, we come closer to one day developing a technique that would enable us to keep the corpus luteum from spontaneously regressing early on in a pregnancy, and in doing so reduce the number of miscarriages. In the animal industry this would greatly reduce economic loss within a herd, and would greatly reduce the emotional devastation many women face upon losing a child. Alternatively, this technique could also be used to create a new estrous synchronization method. This method would utilize the sustained corpus luteum, and its continuous progesterone secretion, by enabling farmers the ability to bring an entire herd into estrus at the same time. As such, continued research into the mechanisms of luteal function is critical to one day developing techniques that would be beneficial to all.

Acknowledgements

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